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# Direct Identification by PCR of EBV Types and Variants in Clinical Samples

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Both Epstein-Barr virus (EBV) type A and type B, and variants of type A, were identified simultaneously by polymerase chain reaction (PCR) amplification of a DNA region coding for a 13 amino acid repeat in the Epstein-Barr virus nuclear antigen (EBNA) 6. Whereas this region varies extensively in type A isolates, no variation was seen in type B isolates. When a repetitive region in the LMP1-coding region was amplified by PCR, it was possible to distinguish individual variants of type B isolates from each other. Forty-two saliva samples from HIV-1–carrying individuals were examined for the presence of type A and type B virus. Both types and multiple variants of each type were found with a much higher frequency than in the saliva samples from healthy individuals. Type A EBV alone was detected in mouthwash samples from 6 infectious mononucleosis (IM) patients. Both type A and B were detected in the peripheral blood B-lymphocytes (PBL) from 1 healthy individual. The same type A variant was demonstrated both in PBL and in the mouthwash sample from another healthy individual. In this study it was shown that a combination of the EBNA 6- and LMP 1-specific PCRs followed by Southern hybridisation can be used to identify both type A and type B virus, as well as to distinguish between multiple variants of the same strain, in saliva and B-cells from both healthy and immunosuppressed individuals. ***J. Med. Virol. 51:355–363, 1997.*** 

**KEY WORDS:** LMPI; EBNA6; AIDS

## INTRODUCTION

Epstein-Barr virus (EBV) is the aetiological agent of the self-limiting lymphoproliferative disorder infectious mononucleois (IM). The virus is also associated with an increasing variety of malignant disorders; for example, undifferentiated nasopharyngeal carcinoma (NPC), endemic Burkitt's lymphoma (BL), and immunoblastic lymphomas of the immunosuppressed are strongly associated with EBV, whereas virus has been detected in 40% of Hodgkin's lymphomas [for review, see Rickinson and Kieff 1996]. Recently EBV (DNA) has been detected in T-cells, adenocarcinoma cells, and smooth muscle cells in human cancers [Sandvej et al., 1994; Imai et al., 1994; McClain et al., 1995]. EBV is also associated with oral hairy leukoplakia (OHL), a lesion found in a proportion of HIV-1–carrying individuals [Walling et al., 1994; Walling and Raab-Traub, 1994]. The virus is tropic for epithelial cells [Sixbey et al., 1984] and B-lymphocytes [Nilsson et al., 1971] in vivo. After primary infection the virus establishes lifelong latency in the host; the virus is found latent in B-cells, and infectious virus has been detected in saliva from both asymptomatic carriers [Sixbey et al., 1984; Yao et al., 1985] and immunosuppressed patients [Sixbey et al., 1989; Kyaw et al., 1992]. EBV is shed from the oropharynx during infectious mononucleosis (IM) [Sixbey et al., 1984] but can also be detected by the polymerase chain reaction (PCR) in the urine from IM patients [Landau et al., 1994]. Replication in the genital mucosa has been suggested [Sixbey et al., 1986].

Two different strains of EBV, designated either A and B (or types 1 and 2) have been described. Each has a worldwide distribution, but type A is more common in Western countries and in Asia, whereas type B seems to be predominant in Africa and New Guinea [Zimber et al., 1986]. These strains can be distinguished from each other on the basis of sequence differences in their EBNA (Epstein-Barr nuclear antigen) 2, 3, 4, and 6 coding regions [Rowe et al., 1989; Sample et al., 1990]. Further distinction between EBV isolates can be made on the basis of mutations affecting different regions of the genome [Walling et al., 1994; Walling and Raab-Traub, 1994; Gratama and Ernberg, 1995].

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Individual isolates of either type A or type B virus can be identified because of the size variation in repetitive elements in the EBNA coding exons that results in proteins of different mobilities on SDS-PAGE; these differences have been exploited in EBNotyping [Gratama et al., 1988; Falk et al., 1995].

It has been demonstrated that isolates from different sites in the same patient are similar on the basis of restriction length fragment polymorphism (RLFP) [Katz et al., 1988]. In the same study it was shown that healthy donors usually harbour the same variant in blood and mouthwash samples. Gratama et al. [1994] have shown previously by EBNotyping that multiple EBV variants can be detected both in immunosuppressed patients and in healthy donors. Human immunodeficiency virus (HIV-1) carriers show higher titers of EBV in mouthwashes and in saliva [Alsip et al., 1988; Lucht et al., 1995] as compared to healthy donors. They frequently harbour type A and type B [Kyaw et al., 1992] viruses simultaneously. Oral hairy leukoplakia (OHL) lesions contain multiple EBV variants [Walling et al., 1994; Walling and Raab-Traub, 1994].

The possible role(s) that strain- or variant-differences may play in disease is not known but clearly requires more detailed epidemiological studies. To this end we have increased the sensitivity, specificity, and simplicity with which variation in EBV can be detected by using a PCR-approach. We have shown previously that a 39 bp repeat in the EBNA 6-coding region varies between 3 and 13 copies, depending on the EBV type A isolate [Falk et al., 1995]. As a complement to the EBNA 6-PCR, we also carried out PCR over a 33 bp repetitive region in the LMP 1 exon, which enabled us to identify virus as type A or type B. In this study we show that a combination of the EBNA 6– and LMP 1–specific PCRs followed by Southern hybridisation can be used to identify both type A and type B virus, as well as to distinguish between multiple variants of the same strain, in saliva and B-cells from both healthy and immunosuppressed individuals.

## MATERIALS AND METHODS

### Cell Lines and Tissue Culture

B95-8 is a marmoset cell line that carry IM-derived EBV [Miller and Lipman, 1973]. Namalva is a Burkitt lymphoma (BL) cell line containing 1 to 2 integrated copies of the EBV genome [Klein et al., 1972]. B95-8, Namalva, D 50, D 9, D 48, D 47, and D 115 all carry type A virus [Falk et al., 1995]. Jijoye M13 [Adldinger et al., 1985], Jijoye p79 [Klein et al., 1978], AG 876 [Dambaugh et al., 1984], Maku [Ehlin-Henriksson et al., 1987], BL 16 [Bernheim et al., 1983], and Silfere [Ehlin-Henriksson et al., 1987], are all BL cell lines that carry type B virus. BL 41 [Rowe et al., 1986] DG75 [Rowe et al., 1986], and K562 [Lozzio and Lozzio, 1975] are all EBV-negative cell lines and were used as negative controls for PCR. All cell lines were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 μg of streptomycin per ml and 60 μg of penicillin per ml. Cells were incubated at 37°C in humidified air that contained 5% $CO_2$.

### DNA Isolation

Standard methods were used to extract DNA from cell lines [Sambrook et al., 1989].

### Collection of Saliva Samples and Mouthwashes from HIV-1–Infected Individuals and Healthy EBV Carriers

Forty-two saliva samples from donors at different clinical stages of HIV-1 infection were used. Unstimulated whole saliva was collected as described by White and Bunting [1936]. Saliva samples were frozen at −70°C within 4 hours of collection. HIV-1–infected individuals were classified according to the criteria of Centers for Disease Control [1992]. Eleven had no clinical signs of infection (CDC group A). Eleven had clinical symptoms corresponding to the AIDS-related complex (ARC; CDC group B) and 20 had full acquired immune deficiency syndrome (AIDS; CDC group C). None of the HIV-1–infected individuals had been treated with acyclovir, foscarnet, ganciclovir, or other anti–herpes virus drugs. Mouthwash samples were collected from IM patients and healthy individuals gargling for 1 minute with 10 ml RPMI 1640 medium [Andersson and Ernberg, 1989]. After removal of bacteria and debris, samples were stored at −20°C. For DNA preparation, 0.1 ml of each mouthwash sample was mixed with 0.1 ml lysis buffer (10 mM Tris-HCL, pH 8.3, 1 mM EDTA, 0.5% NP40, 0.5% Tween-20, and 400 μg/ml proteinase K) and incubated for 1 hour at 55°C. Proteinase K was inactivated by incubation for 15 minutes at 95°C. PCR was undertaken on a 10 μl aliquot of each mouthwash lysate.

IM patients (IM 104, IM 127, IM 133, IM 138, IM 141, IM 146) have previously been found to be EBV-positive, both by EBV-specific serology and the presence of EBV in mouthwashes.

### Purification of CD 19-Positive B-Cells From Healthy Donors

PBLs were isolated from 30 ml blood using the Lymphoprep system as described by the manufacturer (Nycomed, Oslo, Norway). PBLs were washed twice in PBS. Magnetic beads were used to isolate CD-19 positive B-cells according to the manufacturer's instructions (Dynal, Oslo, Norway). Cells were lysed by the addition of 200 μl lysis buffer per $10^6$ B-cells. PCR was carried out on a 10 μl aliquot of each lysate sample.

### PCR-Amplification of a Repetitive Region in the EBNA 6-Coding Region

Samples were amplified by 35 cycles of denaturation (97°C for 60 seconds; first cycle 5 minutes), annealing (52°C for 60 seconds), and extension (72°C for 180 seconds).

PCR reactions (100 μl) contained 20 pmol of each primer (KF 64; 5′ACACTTGAGTTCCATGTCGC3′, co-

ordinates 100,553–100,572 in B95-8 and KF 67 5′TGTAATCACTGGCAAAGGC3′, coordinates 101,198–101,217 in B95-8); 2 mM $MgSO_4$; 10 mM KCl; 20 mM Tris HCl (pH 8.75); 10 mM $(NH_4)_2SO_4$; 1% Triton X-100; 1 mg/ml BSA, 1% glycerol; 200 μM each of dATP, dCTP, dTTP; 160 μM dGTP; 40 μM 7-deaza-dGTP; and 2 U Exo(-) Pfu DNA polymerase (Strategene, La Jolla, CA). PCR products (10 μl aliquots) from the above reactions were reamplified in a volume of 100 μl as described above using the primers KF 65 (5′-TATCGCACGAAGAACAACCCC3′, coordinates 100,585–100,605 in B95-8) and KF 66 (5′AGATGTGGGAACTGGGAGACC3′, coordinates 100,982–101,002 in B95-8) using the following conditions: denaturation (at 97°C for 60 seconds, first cycle 5 minutes), annealing (at 57°C for 60 seconds), and extension (at 72°C for 120 seconds), for a total of 25 cycles.

### PCR-Amplification of LMP 1

Thirty-five cycles of denaturation (94°C for 30 seconds; first cycle 120 seconds), annealing (62°C for 90 seconds), and extension (72°C for 120 seconds) were used for amplification. Reaction mixtures consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0 mM $MgCl_2$, 20 pmole of each primer (LMP 1 s; 5′ACTCTGCTCTCAAAACCTAGGC3′, coordinates 168,592–168,613 in B95-8 and LMP 1 a, 5′ATTTCCAGCAGAGTCGCTAG3′, coordinates 168,373–168,392 in B95-8), and 200 μM each of dATP, dGTP, dCTP, dTTP, 2.5 U Taq polymerase (Perkin-Elmer), 0.5% Tween-20, and 0.5% NP-40.

### Detection of EBV by Hybridisation

PCR products were separated on 1.5% agarose gels and a standard protocol for hybridisation was used [Falk et al., 1995]. Hybridisation was carried out at 50°C (except for the type A probe, where 65°C was used) using end-labeled oligonucleotide probes. Four different oligonucleotides were used for detection of amplified fragments. To simultaneously detect types A and B, a 20 nt probe (5′CAACCCCGGTATGAGGATCC3′) corresponding to basepairs 100,599–100,618 in B95-8 was used. The type A–specific probe (5′CTCGACCAACTCGTCGTTGC3′) corresponded to base pairs 100,652–100,671 in the B95-8 genome, and the type B–specific probe (5′TCGGCTCAATGGGAGCCACA3′) corresponded to the same region of AG 876. For the detection of LMP 1, a 33 nt probe (5'GTCATCAGTGTTGTCAGGGTCCTGTGGGCCATT3') that corresponds to coordinates 168,505–168,537 of B95-8 was used. Primers were synthesized by Pharmacia (Uppsala, Sweden).

### Nucleotide Sequencing of the LMP 1 33 bp Repeat

Samples for sequencing were first amplified by PCR: the primers used were LMP 1s and LMP 1a. Sequencing was carried out using a dye-terminator cycle sequencing core kit according to the manufacturer's instructions (Perkin Elmer, Foster City, CA). DNA was sequenced using LMP 1s as primer. Samples were subjected to electrophoresis using an automated sequencer (Applied Biosystems).

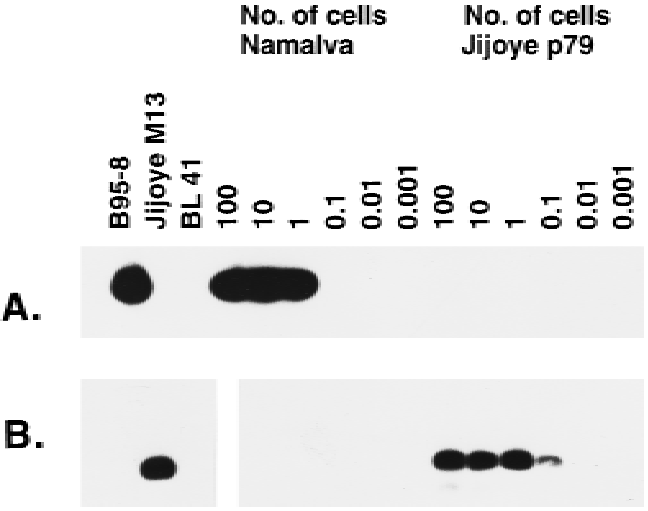


Fig. 1. Assessment of the sensitivity of PCR-amplification of the 39 bp repeat sequence in the EBNA 6 coding BERF 4 exon. The sensitivity was tested by applying the PCR. Nested PCR followed by hybridisation with probes **(A)** specific for EBV type A, and **(B)** specific for type B, where performed on 10-fold dilutions of Namalva cell-lysates and Jijoye p79, as described in Materials and Methods. The Namalva cell line contains 1–2 EBV genomes/cell and the Jijoye p79 cell line contains on average 11 genomes. B95-8, Jijoye M13, and BL 41 were used as positive and negative controls.

## RESULTS

### Sensitivity and Specificity of PCR-Amplification of the EBNA 6 39 bp Repeat

We have used previously PCR-amplification of the 39 bp repeat in the EBNA 6 BERF 4 exon to distinguish between type A isolates in established cell lines that carried EBV of different donor origins [Falk et al., 1995]. Before using this assay directly on patient material it was necessary to establish the sensitivity of the nested-PCR reaction. Tenfold dilutions of DNA extracted from Namalva cells and from Jijoye p79 cells were subjected to PCR. The BL-derived Namalva line contains 1–2 EBV genomes/cell and carries type A virus. Jijoye p79, a subline of Jijoye [Klein et al., 1978], carries the type B virus genome, and no virus is produced as determined by immunofluorescence staining of early and viral capsid antigens (data not shown). Nested-PCR followed by hybridisation were carried out as described in Materials and Methods. With the type A probe a specific signal was obtained with DNA from a single Namalva cell, which suggests that every genome present can be detected (Fig. 1a). No signal was obtained for type B virus (Jijoye p79) when the type A–specific probe was used, confirming probe specificity. The sensitivity of the type B–specific probe was also established; this probe gave a positive reaction with 0.1 cells. Jijoye p79 carries on average 11 genomes per cell [Tanaka et al., 1976; Klein et al., 1978], indicating that the type B–specific probe detected 1 copy of the genome

TABLE I. Detection of EBV Type A and Type B by PCR

| Sample* | Percentage of total cases tested in PCR | | | Percentage of type distribution of the positives | | | Multiple variants of type A |
|---|---|---|---|---|---|---|---|
| | Total | Pos, % | Neg, % | A, % | A and B, % | B, % | |
| HIV-1 positive individuals | 42 | 74 (31/42) | 26 (11/42) | 48 (15/31) | 23 (7/31) | 29 (9/31) | |
| Asymptomatic | 11 | 64 (7/11) | 36 (4/11) | 29 (2/7) | 29 (2/7) | 43 (3/7) | 0 |
| ARC (incl. OHL) | 17 | 76 (13/17) | 24 (4/17) | 54 (7/13) | 23 (3/13) | 23 (3/13) | 4 |
| AIDS (incl. OHL) | 14 | 79 (11/14) | 21 (3/14) | 55 (6/11) | 18 (2/11) | 27 (3/11) | 2 |
| HIV-1 infected individuals with OHL (ARC and AIDS) | 12 | 83 (10/12) | 17 (2/12) | 60 (6/10) | 30 (3/10) | 10 (1/10) | 2 |
| Healthy donors | 16 | 69 (11/16) | 31 (5/16) | 82 (9/11) | 0 | 18 (2/11) | 2 |
| Healthy donors CD 19+(B-cells) | 4 | 75 (3/4) | 25 (1/4) | 67 (2/3) | 33 (1/3) | 0 | 1 |
| Mono | 6 | 100 (6/6) | 0 | 100 (6/6) | 0 | 0 | 2 |

* = mouthwashes, except CD19+healthy donor

and that sensitivity was comparable to that of the type A–specific probe (Fig. 1B). No signal was obtained for the Namalva cell line (type A virus) when the type B–specific probe was used. Hence both probes were highly sensitive and specific.

### Sensitivity and Specificity of PCR-Amplification of the LMP 1 33 bp Repeat

To distinguish between type B isolates, we performed PCR-amplification of the 33 bp repeat in the LMP 1–coding region, because of the variation in number of repeats between isolates. Tenfold dilutions of DNA extracts of both Namalva and Jijoye M13 were used to establish the sensitivity and specificity of PCR amplification (for details, see Materials and Methods). The method was highly sensitive, capable of detecting 0.5–5 copies of the EBV genome (data not shown). EBV-negative cell lines (used as negative controls) examined at the same time were all negative (data not shown), as expected.

### Detection of Types A and B, and Variants Thereof, in Saliva Samples from HIV-1–Positive Individuals

We have shown previously that the above PCR products can be used both to distinguish between EBV-variants according to size and to distinguish between the major EBV types, when hybridised consecutively with probes that either detect type A and type B simultaneously or type B alone [Falk et al., 1995]. We have now developed a probe specific for type A, i.e., that does not cross-react with type B virus (Fig. 1A). With these methodologies a total of 42 samples from HIV-1–positive individuals were analysed, 12 of whom had OHL. Thirty-one (74%) HIV-1–positive individuals secreted EBV in their saliva, as evaluated by EBNA 6–specific PCR. Of these 48% were positive for type A virus alone, 23% were positive for both type A and type B, and 29% were positive for type B virus alone (Figs. 2 and Fig. 3; Table I). For the group of HIV-1–infected asymptomatic individuals group (n = 7), 2 carried type A virus alone, 2 had dual carriage, and 3 carried type B virus alone (Table I; Figs. 2 and 3), as determined by

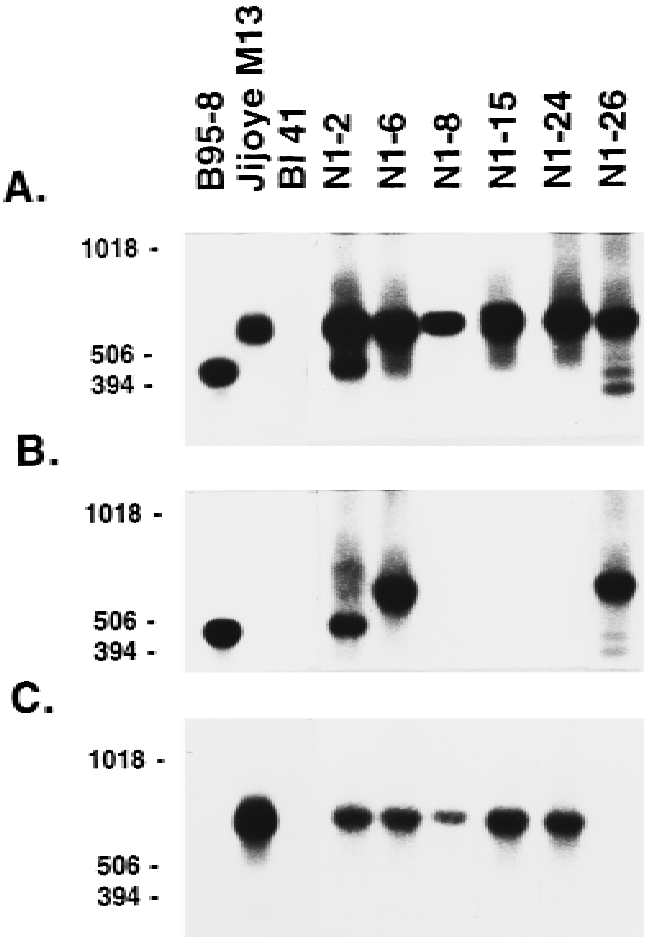


Fig. 2. Detection of EBV-DNA in saliva samples from six HIV-1–infected individuals by PCR-amplification of the EBNA 6 39 bp repeat sequence. N1-2, N1-6, and N1-8 were asymptomatic, N1-15 had ARC, and N1-24 and N1-26 had AIDS. Nested PCR-amplification of the 39 bp repeat, followed by hybridisation with probes **(A)** specific for EBV type A and B, **(B)** specific for type A, and **(C)** specific for type B were performed as described in Materials and Methods. B 95-8, Jijoye M13, and BL 41 were used as positive and negative controls. Sizes (bp) of the molecular weight markers are indicated to the left.

EBNA 6–specific PCR of saliva. For the group of individuals with ARC (n = 13), 7 carried type A virus alone, 3 carried both types, and 3 had type B virus

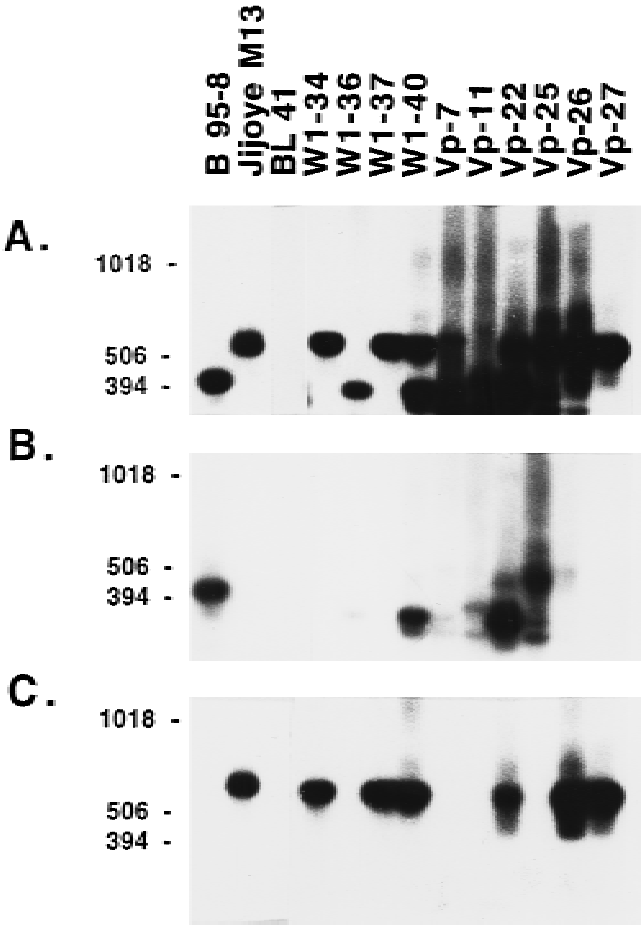


Fig. 3. Detection of EBV-DNA in mouthwash samples from ten HIV-1 carriers by PCR amplification of the EBNA 6 39 bp repeat sequence. All patients had ARC except Vp 26, who had AIDS. Nested PCR-amplification of the 39 bp repeat, followed by hybridisation with probes **(A)** for both EBV type A and B, **(B)** specific for type A, and **(C)** specific for type B were as described in Materials and Methods. B 95-8 was used as a positive control, and Jijoye M13 and BL 41 were negative controls. Sizes (bp) of molecular weight markers are indicated to the left.

alone. For those individual with AIDS (n = 11), 6 carried type A alone, 2 carried both type A and type B, and 3 carried type B alone. For HIV-1–infected symptomatic individuals (ARC and AIDS), multiple variants of type A were identified, as judged by the presence of multiple specific-PCR products of different sizes in 6 of 31 positive patients. Six HIV-1 infected individuals with OHL carried type A virus, 3 carried both types, and 1 carried type B only. Because the 39 bp repeat in type B isolates did not vary in size (Figs. 2C and 3C), the assay could not be used to distinguish between type B variants. We sequenced two type B–carrying cell lines (AG 876 and Jijoye M13), and in both cases only 1 degenerate copy of the 39 bp repeat was detected (data not shown). Therefore, to distinguish between type B isolates, we took advantage of the size variation in the 33 bp repeat in the LMP 1 C-terminal region (see below).

### Mouthwashes From Healthy Individuals Contained Only Type A or Type B EBV

The EBNA 6–specific PCR was used to examine 16 mouthwash samples from healthy individuals for the presence of type A and type B virus (Table I). Sixty-nine percent were positive for EBV. Nine of the positive samples contained type A virus–specific sequences, and 2 carried type B-specific sequences. Multiple variants of type A virus were detected in 2 samples.

### EBV Variants Present in Peripheral Blood and Mouthwash From the Same Healthy Individual Were Identical

We purified B-cells (see Materials and Methods for details) from 4 healthy donors and 1 sample was found to be negative for EBV in our assay. Two carried type A virus, while the third carried both types A and B (Fig. 4). One individual had same type A variant in both mouthwash and B-cells (H3, Fig. 4). One individual had 2 variants of type A EBV in B-cells (data not shown).

### Type A EBV Alone Was Present in Mouthwash Samples from IM Patients

A total of 6 mouthwashes IM patients were subjected to EBNA 6–specific PCR. All carried type A virus but were negative for type B virus. Two mouthwashes contained multiple variants (Fig. 5).

### Variation in the Size of the LMP 1 Protein Correlated With Variation in the Size of the PCR-Amplified 33 bp Repeat Region

We analysed a total of 11 isolates comprising 6 cell lines that carry type A virus and 5 cell lines that carry type B virus. We performed PCR over the 33 bp repeat region in LMP. It was found that the size of the PCR product varied between different isolates (Fig. 6A). The observed size variation in the 33 bp repeat region correlated with the size variation in the protein as determined by Western blotting (data not shown). Therefore we compared the DNA sequences of the products and confirmed that the size variation correlated with the number of repeats of the 33 bp sequence. The number of repeats varied from 3 to 5, with some additional insertions occasionally present (Fig. 6B for details).

### The 33 bp Repeat in the LMP1-Coding Region Was Used to Distinguish Between Different Isolates of EBV-Type B in Saliva

Saliva samples from 13 HIV-1–positive individuals harbouring type B virus were examined by LMP 1–specific PCR. With this method, multiple variants of type B virus were detected in 6 patients as indicated by the multiple bands obtained (Fig. 6C; patients: N1–2, W1–16, W1–37, W1–34, Vp-22, Vp-27).

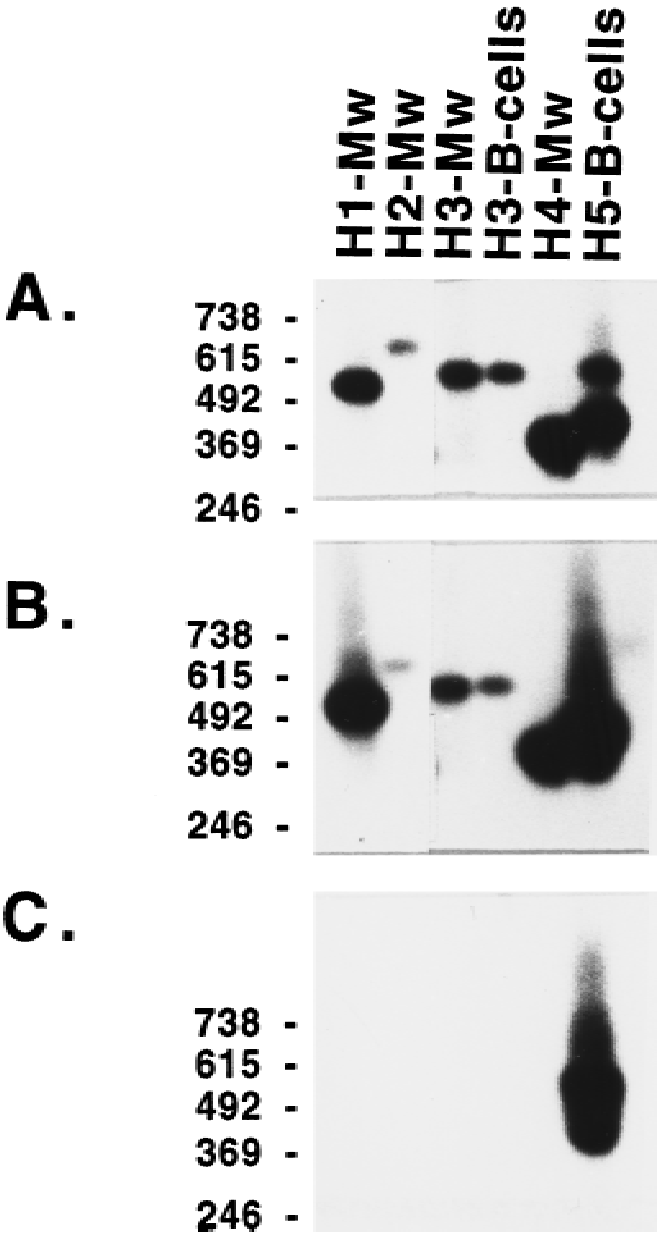


Fig. 4. Detection of EBV-DNA in mouthwash samples (H1-Mw, H2-Mw, H3-Mw, H4-Mw) and CD19-enriched B cells from healthy EBV-seropositive individuals (H3-B-cells, H5-B-cells) by PCR-amplification of the EBNA 6 39 bp repeat sequence. H3-Mw and H3-B-cells are from the same individuals. Nested PCR-amplification of the 39 bp repeat as in Figure 1, followed by hybridisation with probes **(A)** for both EBV types A and B, **(B)** specific for type A, and **(C)** specific for type B. Sizes (bp) of molecular weight markers are indicated to the left. The same controls as in Figure 2 were used both in the PCR assay and hybridisation.

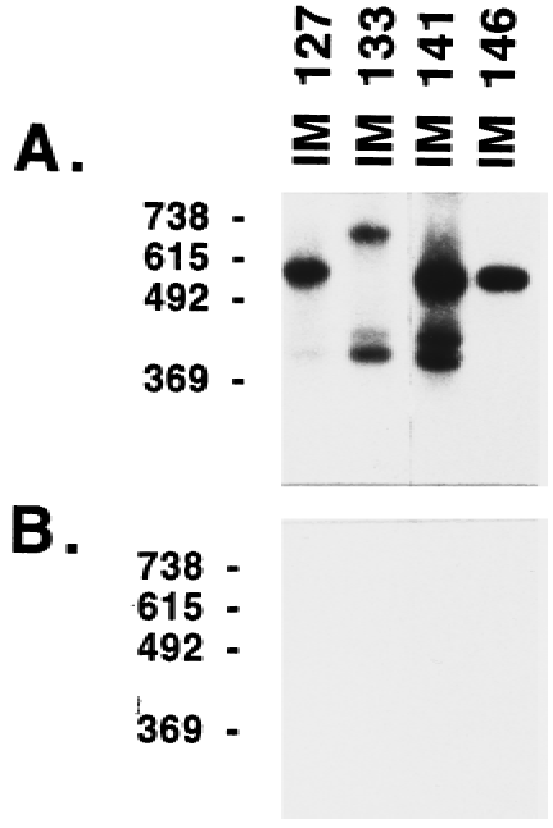


Fig. 5. Detection of EBV-DNA in mouthwash samples from 4 patients with acute infectious mononucleosis by PCR-amplification of the EBNA 6 39 bp repeat sequence. Nested PCR over the n × 39 bp repeat as in Figure 1, followed by hybridisation with a probe **(A)** specific for type A and **(B)** specific for type B. The relevant sizes (bp) of the molecular weight markers are indicated to the left. Type A- and type B-specific controls were used in the PCR assay and hybridisation as described in the legend to Figure 2.

## DISCUSSION

We developed a sensitive method that makes it possible to distinguish between EBV types A and B as well as between different variants of both types A and B. Amplification of a 39 bp repeat sequence in EBNA 6 using nested PCR followed by Southern hybridisation allowed identification of EBV as either type A or B. Variation in the number of repeats of the 39 bp sequence in EBNA 6 allowed identification of different isolates of type A. In contrast, we could not distinguish between different type B isolates using this approach, as only a single partially homologous copy of this repeat is present. Therefore the EBNA 6–specific PCR was complemented by using PCR-amplification of the 33 bp repeat sequence in the LMP 1–coding region. Although several combinations of non-nested PCR have been employed [Lin et al., 1993; Sample et al., 1990], the approach which we describe is the first to allow identification of different isolates of EBV directly in clinical samples. In order to test our PCR-approach, the following clinical samples were examined: saliva samples from HIV-1–positive individuals, mouthwash samples from healthy individuals, mouthwash samples from IM patients, and B-cells from healthy individuals.

In 6 mouthwash samples from IM patients, type A EBV alone was detected. In 2 of these IM patients, multiple bands were detected, thus indicating recombination during the primary infection. These data, together with other reports using EBNA 2–based PCR, suggest that IM rarely, if ever, is caused by type B EBV [for review, see Gratama and Ernberg, 1995]. Similar results were obtained by Sandvej et al. [1994] in a

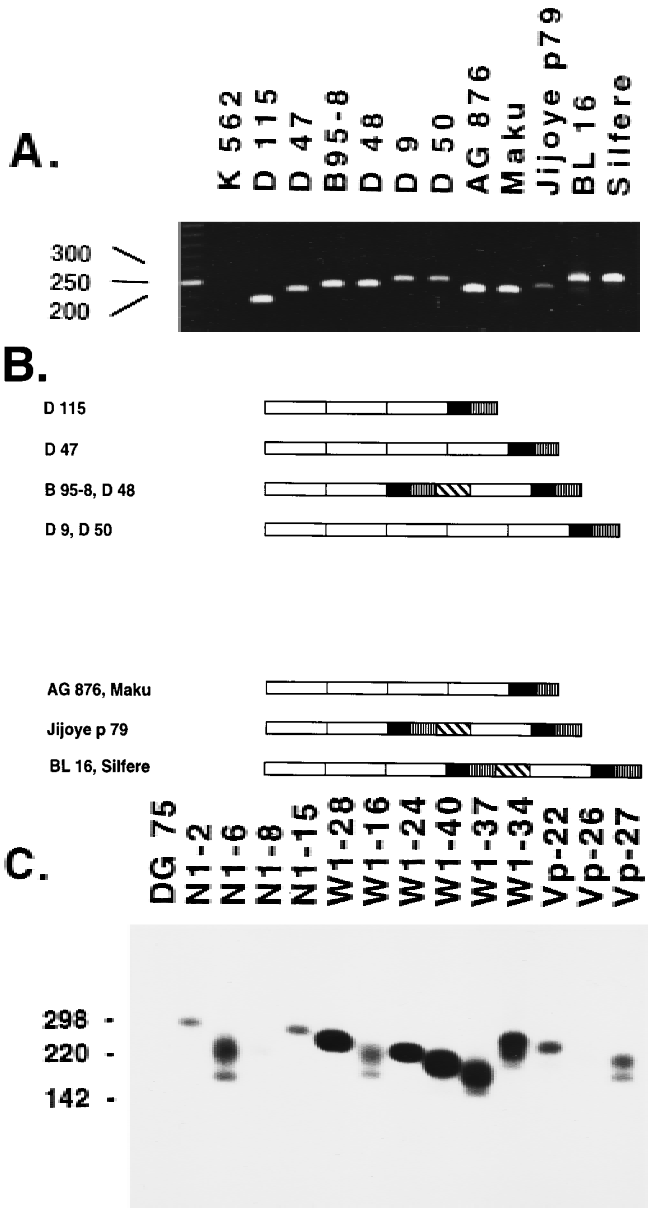


Fig. 6. PCR-amplification of a 33 bp repeat in LMP1 can be used to distinguish between EBV isolates. **(A)** Size variation in PCR products obtained after amplification of the 33 bp repeat, followed by gel electrophoresis and visualisation of products by ethidium bromide staining as described in Materials and Methods. Six cell lines carrying type A virus (D 115, D47, B95-8, D 48, D 9, D 50) and five cell lines carrying type B virus (AG 876, Maku, Jijoye p79, BL 16, Silfere) were analysed. Sizes (bp) of molecular weight markers are indicated to the left. **(B)** A schematic representation of the organisation of the LMP-repeat in PCR-products as determined by sequencing. The white boxes represents a complete 33 bp repeat, the black boxes represents 12 bp, the vertical striped boxes represents 15 bp, and the diagonally striped boxes represent an incomplete repeat consisting of 21 bp. **(C)** PCR followed by hybridisation with an oligonucleotide specific for LMP1, of DNA from 13 saliva samples from HIV-1-carriers, all with EBV type B. Sizes (bp) of molecular weight markers are indicated to the left. DG 75 and K 562 were used as negative controls.

study of Danish patients. This study also showed that type A was present in Hodgkin's disease and infectious mononucleosis, and in both Danish and Malaysian patients with peripheral T-cell lymphomas.

Type A EBV was clearly predominant in the throat washings of EBV sero-positive individuals from Japan, who were either apparently in good health or had tonsillitis [Kumimoto et al., 1992]. We verified previous observations, made with less sensitive techniques, that healthy carriers usually secrete only one EBV type in the saliva. Both Yao et al. [1991] and Sixbey et al. [1989] examined throat washings for the presence of type A and type B and found dual carriage in a minority of the cases. Six paired washings from throat and cervix showed that type B virus is more common in the cervix and that dual carriage occurs [Sixbey et al., 1989]. We detected both type A and type B in B-cells from a healthy individual. Previous studies to determine if both type A and B coexist in B-cells have relied on establishing lymphoblastoid cell lines (LCLs) [for review, see Gratama and Ernberg, 1995]. Type B virus has a much lower transforming efficiency than type A, and therefore detection of type B EBV may be underrepresented in such studies.

It has been reported previously that HIV-1–infected patients secrete more EBV as compared to amount secreted by healthy individuals [Alsip et al., 1988; Lucht et al., 1995]. We were unable to detect virus in 33% of the saliva samples obtained from HIV-1–positive individuals. Twenty-nine percent of the samples from HIV-1–positive persons carried only type B virus and approximately 25% had dual carriage. Sixbey et al. [1989] detected dual carriage in 50% of mouthwash samples from HIV-1 carriers and 20% of these carried only type B.

In our study, 60% of individuals with OHL carried type A virus. Kyaw et al. [1992] found a much lower frequency of type A EBV when they analysed throat washings from individuals with OHL by using PCR-amplification of the EBNA-2 gene. The differences may reflect different geographic distributions of type B, since the material in the study performed by Kyaw et al. [1992] was collected in Sydney, Australia. Taken together, these studies suggest that the type B strain is more common among HIV-1–infected individuals and that the incidence of type B and dual carriage is not significantly higher in individuals with OHL as compared to those individuals with HIV-1 but without OHL.

The extensive polymorphism in EBV genotypes allows the use of RFLP to track viral transmission patterns along natural and iatrogenic (e.g., organ transplantation) routes. EBV offers unique possibilities to understand basic aspects of virus-host interactions, because the virus is widespread and establishes lifelong latency with a strong and well-developed immunosurveillance [Masucci and Ernberg, 1994]. EBV is involved in a wide variety of pathogenic processes. Our understanding of cellular and humoral control mechanisms is increasing.

Central questions to be addressed are: how frequently and by what mechanism does EBV undergo genetic drift in vivo? What are the consequences of immune surveillance and thus disease outcome? What are the interactions between different cell types, i.e., epithelial and lymphoid, that harbour EBV? Can virus be

transferred from one cellular compartment to another? In order to answer these questions, it is important to be able to detect viral variants not only in the population in general, but also within the individual. The approach we describe above provides a simple and sensitive way to address these questions.

Epidemiological studies employing methods to distinguish between different virus isolates suggested that as a rule, healthy carriers harbour 1 EBV variant [for review, see Gratama and Ernberg 1995]. It seems that an intact immune response to the virus prevents superinfection by exogenous virus. Superinfection is probably a regular threat, because the virus is ubiquitous, and symtom-free virus carriers frequently secrete biologically active virus. Nevertheless, multiple virus variants have been identified among isolates from approximately 15% of healthy carriers [Gratama et al., 1994]. In one case, 14 different variants were identified in the same individual. In some cases, these variants were the result of changes in the molecular weight of only one EBNA-protein as determined by immunoblotting (EBNotyping). We have suggested that these variants arise because of recombination between endogenous EBV genomes during latency, or more likely, during lytic multiplication in the oropharynx. Superinfection appears to be rare in healthy donors because there have been few reports of both type A and type B in healthy individuals.

In contrast, for immunosuppressed individuals, several studies have shown dual carriage of type A and type B, as well as the presence of multiple variants as determined by EBNotyping [for review, see Gratama and Ernberg, 1995]. In cases where the molecular weights of most EBNAs varied, it is likely that the presence of several variants in the same patient is due to superinfection because of breakdown of immunological barriers. In immunosuppressed patients, this may also relate to increased replication of endogenous EBV because of a more relaxed immunoregulation of latency. This in turn may speed up generation of new variants by recombination between endogenous and superinfecting viral genomes. The PCR-methodology described above allows both rapid and specific detection of EBV variants, which will allow further analysis of this virus-host interaction.

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